# Carboxyl terminus of apolipoprotein A-I (ApoA-I) is necessary for the transport of lipid-free ApoA-I but not prelipidated ApoA-I particles through aortic endothelial cells

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Abstract: High density lipoproteins (HDL) and apolipoprotein A-I (apoA-I) must leave the circulation and pass the endothelium to exert their atheroprotective actions in the arterial wall. We previously demonstrated that the transendothelial transport of apoA-I involves ATP-binding cassette transporter (ABC) A1 and re-secretion of lipidated particles. Transendothelial transport of HDL is modulated by ABCG1 and the scavenger receptor BI (SR-BI). We hypothesize that apoA-I transport is started by the ABCA1-mediated generation of a lipidated particle which is then transported by ABCA1-independent pathways. To test this hypothesis we analyzed the endothelial binding and transport properties of initially lipid-free as well as prelipidated apoA-I mutants. Lipid-free apoA-I mutants with a defective carboxyl-terminal domain showed an 80% decreased specific binding and 90% decreased specific transport by aortic endothelial cells. After prior cell-free lipidation of the mutants, the resulting HDL-like particles were transported through endothelial cells by an ABCG1- and SR-BI-dependent process. ApoA-I mutants with deletions of either the amino terminus or both the amino and carboxyl termini showed dramatic increases in nonspecific binding but no specific binding or transport. Prior cell-free lipidation did not rescue these anomalies. Our findings of stringent structure-function relationships underline the specificity of transendothelial apoA-I transport and suggest that lipidation of initially lipid-free apoA-I is necessary but not sufficient for specific transendothelial transport. Our data also support the model of a two-step process for the transendothelial transport of apoA-I in which apoA-I is initially lipidated by ABCA1 and then further processed by ABCA1-independent mechanisms.

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# The Carboxy-Terminus of Apolipoprotein A-I (ApoA-I) is necessary for the Transport of lipid-free ApoA-I but not pre-lipidated ApoA-I Particles through aortic endothelial Cells

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Running title: Transendothelial ApoA-I Transport: a two-step Process

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**High density lipoproteins (HDL) and apolipoprotein A-I (apoA-I) must leave the circulation and pass the endothelium to exert their atheroprotective actions in the arterial wall. We previously demonstrated that the transendothelial transport of apoA-I involves ATP-binding cassette transporter (ABC) A1 and re-secretion of lipidated particles. Transendothelial transport of HDL is modulated by ABCG1 and the scavenger receptor BI (SR-BI). We hypothesize that apoA-I transport is started by the ABCA1-mediated generation of a lipidated particle which is then transported by ABCA1-independent pathways. To test this hypothesis we analysed the endothelial binding and transport properties of initially lipid-free as well as pre-lipidated apoA-I mutants. Lipid-free apoA-I mutants with a defective carboxy-terminal domain showed an 80% decreased specific binding and 90% decreased specific transport by aortic endothelial cells (ECs). After prior cell-free lipidation of the mutants the resulting HDL-like particles were transported through ECs by an ABCG1-dependent but SR-BI-independent process. ApoA-I mutants with deletions of either the amino-terminus or both the amino- and carboxy-terminus showed dramatic increases in non-specific binding and transport but no specific binding or transport. Prior cell-free lipidation did not rescue these anomalies. Our findings of stringent structure-function relationships underline the specificity of transendothelial apoA-I transport and suggest that lipidation of initially lipid-free apoA-I is necessary but not sufficient for specific transendothelial transport. Our data also support the model of a two-step process for the transendothelial transport of apoA-I in which apoA-I is initially lipidated by ABCA1 and then further processed by ABCA1-independent mechanisms.**

Atherosclerosis is a progressive disease which is characterized by lipid accumulation in macrophages in the arterial wall and leads to complications like heart attacks and strokes (1). Low plasma levels of high density lipoprotein (HDL) cholesterol as well as apolipoprotein A-I (apoA-I) are associated with increased risk of coronary heart disease (2).

Mature HDL particles are synthesized mainly in the liver and intestine by a multi-step process which is initiated by the binding of cellular phospholipids and cholesterol to initially lipid-free apoA-I. This step requires the presence of the ATP-binding cassette transporter (ABC) A1

(3). Based on X-ray crystallography and computer modelling, most of the 243 amino acid residues of apoA-I are grouped in amphipathic alpha-helices, 11 or 2 × 11 amino acids in length (4-10), that embrace the carbon chains of several phospholipid molecules like a belt.

Both HDL and apoA-I were found to exert multiple anti-atherogenic properties, for example on the function and viability of the endothelium, the cholesterol homoeostasis and inflammatory state of macrophages, lipoprotein oxidation, coagulation, and thrombosis (2,11). One major anti-atherogenic effect is the removal of excess cholesterol from macrophage foam cells of atherosclerotic lesions and its delivery to the liver for biliary excretion (12).

To fulfill their atheroprotective actions in the subendothelial space of arteries, HDL or its precursor, lipid-poor apoA-I, have to leave the circulation and pass the endothelium. This cellular monolayer of the interior surface of blood vessels forms a semi-permeable barrier and regulates liquid and solute transport between intra- and extravascular compartments (13). Little is known on how plasma proteins including HDL and apoA-I cross this endothelial barrier and enter the vascular wall (14). Morphological, biochemical, and physiological studies have suggested both paracellular and transcellular transport of proteins through the intact endothelium. We previously demonstrated that the transendothelial transport of both HDL and apoA-I involves saturable and specific processes. Transport of HDL is modulated by ABCG1 and the scavenger receptor BI (SR-BI) (15) whereas the transendothelial transport of lipid-free apoA-I is modulated by ABCA1.

After apical-to-basolateral transport though aortic endothelial cells (ECs) the initially lipid-free apoA-I was recovered as a lipidated particle (16-17). From this observation and in analogy to similar observations on apoA-I and ABCA1 mediated lipid efflux from macrophages (18-20), we hypothesize that the transendothelial transport of apoA-I is a two-step process. First a functional interaction between apoA-I and ABCA1 is required to generate a lipidated particle which is subsequently transported by ABCA1 independent processes. To test this hypothesis, we investigated the endothelial binding and transport properties of initially lipid-free as well as pre-lipidated apoA-I mutants which have been previously well characterized for their capacity to induce ABCA1-dependent phospholipid and cholesterol efflux from macrophage cell lines and to form HDL both in vitro and in vivo (21). Specifically we compared recombinant wild-type (WT) apoA-I, two mutants with either a deletion (apoA-I(Δ185-243)) or multiple amino acid substitutions in the carboxy-terminal domain, which are both defective in eliciting ABCA1 mediated lipid efflux from macrophages, as well as three apoA-I mutants with deletions of either the amino-terminus (apoA-I(Δ1-59)) or midregional domains (apoA-I(Δ144-165)) or both the amino- and carboxy-terminal domains (apoA-I(Δ1-59/Δ185-243)), which have no or only slightly impaired lipid efflux capacity (22). Our data show that the carboxy-terminal ABCA1 interaction domain of apoA-I is mandatory for the transendothelial transport of lipid-free apoA-I but not of pre-lipidated apoA-I particles and thus support the model of a two-step process for transendothelial apoA-I transport.

## Experimental Procedures

**Cell culture -** ECs were isolated from bovine aortas as described previously (23) and cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with 5% fetal calf serum (FCS) at 37°C in a humidified 5% $CO_2$, 95% air incubator.

**Small interfering (si)RNA transfection -** ECs were transfected with 100 nM Stealth small interfering (si)RNA against either ABCA1, ABCG1, ABCA1/ABCG1 or SR-BI and 34 nM BLOCK-iT fluorescent oligo (Invitrogen) as described previously (15). Binding and transport assays were conducted between 65 and 72 h after transfection. The efficiency of the silencing was evaluated by quantitative RT-PCR and Western blotting, as shown previously (15,17).

**Isolation of HDL and apoA-I from plasma -** Human HDL (1.063<d<1.21 kg/l) was isolated from fresh normolipidemic plasmas of blood donors by sequential ultracentrifugation (24). The purity of the lipoprotein preparation was verified by SDS polyacrylamide gel electrophoresis (SDS-PAGE) in order to assure no contamination with LDL or albumin. Lipid-free human plasma WT apoA-I was further purified from delipidated HDL as described previously (25)

**Production and isolation of recombinant apoA-I -** Production of the recombinant WT apoA-I and of the apoA-I mutants Δ(185-243),

Δ(144-165), Δ(1-59), and Δ(1-59)/Δ(185-243) was described previously (26), (22), (27), (28), (29).

Generation of adenoviruses expressing the apoA-I(L218A/L219A/V221A/L222A): The apoA-I gene lacking the BglII restriction site (that is present at nucleotide positions 181 of the genomic sequence relative to the ATG codon of the gene), was cloned into the pCDNA3.1 vector to generate the pCDNA3.1-apoA-I(ΔBglII) plasmid as described (30). This plasmid was used as a template to introduce the apoA-I(L218A/L219A/V221A/L222A) mutations in apoA-I using the mutagenesis kit QuickChange® XL (Stratagene) and the mutagenic primers. The forward (F) and reverse (R) primers used are F: 5'GGACCTCCGCCAAGGCGCGGCGCCCGCGGCGGAGAGCTTCAAGGTC-3' and R: 5'-GACCTTGAAGCTCTCCGCCGCGGGCGCCGCGCCTTGGCGGAGGTCC-3' (sites of the mutagenesis are underlined). Following 18 cycles of PCR amplification of the template DNA, the PCR product was treated with DpnI to digest plasmids containing methylated DNA in one or both of their strands. The reaction product consisting of plasmids containing newly synthesized DNA carrying the mutations of interest were used to transform competent XL-10 blue bacteria cells (Stratagene). Ampicillin-resistant clones were selected, and plasmid DNA was isolated from these clones and subjected to sequencing to confirm the presence of the point mutations. The 2.2 kb apoA-I inserts containing the apoA-I mutant was cloned into the pAdTrack CMV vector which was used to generate the adenoviral constructs by recombination with the Ad-Easy-1 helper virus in the bacteria cells BJ-5183-pAD1(Stratagene), that contain the Ad-Easy-1 helper virus. Correct clones were propagated in DH5a bacteria cells. The recombinant adenoviral constructs were linearized after incubation with PacI and used to transfect 911 cells. Following large-scale infection of human embryonic kidney 293 cell cultures, the recombinant adenoviruses were purified by two consecutive CsCl ultracentrifugation steps, dialyzed and titrated. The mutant protein was isolate from the culture medium of HTB-13 cells infected with the apoA-I(L218A/L219A/V221A/L222A) expressing adenovirus as described (22,26).

Lyophilized apoA-I mutants were dissolved in 5 M Guanidiniumhydrochloride and dialysed against 0.01 M Tris-HCl pH 8, 0.15 M NaCl. The iodination was carried out by the same procedure as described before for WT apoA-I adjusted to pH 8 and the extensive dialysis was against 0.01 M Tris-HCl pH 8, 0.15 M NaCl.

**Radiolabeling of apoA-I -** ApoA-I was labeled with $^{125}$I using the Iodo-Beads iodination reagent (Pierce) and Na$^{125}$I (Hartmann Analytic) according to the manufacturer's instructions. In a typical reaction, we used 0.5 mCi of Na$^{125}$I, 0.7 mg of apoA-I, and two beads. Protein was separated from unincorporated $^{125}$I with a Sephadex G-25 column (Amersham Biosciences) followed by extensive dialysis (against 0.15 M NaCl, 0.3 mM EDTA, pH 7.4) to remove residual free iodine. The specific activity expressed as cpm/ng protein was calculated based on the protein concentration measured by the DC protein assay (Bio Rad) and the activity measured using a γ-counter (Perkin Elmer). Specific activities of 600–1200 cpm/ng protein were obtained.

**Preparation of reconstituted HDL (rHDL) -** Discoidal rHDL particles were produced by the cholate dialysis method (18) and contained WT or mutant apoA-I, 2-oleoyl-1-palmitoyl-sn-glycero-3 phosphocholine (POPC) (Sigma), and sodium cholate (Sigma) in a molar ratio of 1/40/100 ((mutant) ApoA-I 1:40). Reconstituted HDL was iodinated as described above for apoA-I. Electron microscopy analysis of the particles was performed as described (22).

**Binding and cell association assays -** Binding and cell association assays with $^{125}$I-apoA-I or $^{125}$I-rHDL were performed as described previously (16). ECs were incubated with the indicated concentrations of $^{125}$I-apoA-I or $^{125}$I-rHDL without (total) or with (non-specific) a 40-fold excess of the indicated competitor for 2 h at 4°C (binding) or 1 h at 37°C (cell association). Specific binding/cell association was calculated by subtracting the values of non-specific binding/cell association from those of total binding/cell association. All experiments were performed at least as triplicates.

**Transport assays -** Transport assays were performed as previously described (16). In brief, ECs were seeded 2 days in advance on inserts (0.4 μm, BD Biosciences) precoated with collagen type I (BD Biosciences). Medium containing either $^{125}$I-apoA-I or $^{125}$I-rHDL at the indicated concentrations was added to the apical compartment together with (non-specific transport) or in the absence (total transport) of a 40-fold excess of unlabeled apoA-I and rHDL,

respectively. After incubation for 60 min at 37°C the media of the basolateral compartment were collected to measure radioactivity. Specific transport was calculated by subtracting the values of non-specific transport from those of total transport.

**Gel filtration chromatography -** The size of apoA-I and mutant apoA-I before and after transendothelial transport was analyzed by gel filtration chromatography as described (31). In brief transport assays were performed in 4 transwell cell culture wells as described above. The media (2 ml) were isolated from the basolateral compartments, combined and concentrated to 50 µl by centrifugal concentrators (Vivaspin 500, Sartorius Stedim Biotech). The concentrate was loaded onto a Superdex$^{TM}$ 200 prep grade HiLoad$^{TM}$ 16/60 column (GE-Healthcare) of an Akta fast-protein liquid chromatography (FPLC) system and eluted with Tris saline (0.01 M Tris, 0.15 M NaCl, 0.1 mM EDTA, pH 7.5) at a flow rate of 1.5 mL/min. Fractions (0.5 mL) were collected and the amounts of transported radioactivity were determined using a Perkin Elmer γ-counter.

**Native agarose gel electrophoresis** - Equal amounts of proteins were used for mobility analysis and were loaded on a 1% native agarose gel (0.05 M barbital buffer, pH 8.6). Electrophoresis was performed at 4°C. After that, the gel was stained with Coomassie (protein staining) and Sudan black (lipid staining) using standard protocols.

**Statistical analyses -** The data for all experiments were analyzed using GraphPad Prism 5 software program. Comparisons between groups were performed using t-test methods. Experiments were routinely performed in triplicates or quadruplets. Each experiment shown is a representative of at least three similar experiments. If not indicated otherwise the data are graphically represented as means +/- SD.

## Results

**Binding and association of lipid-free apoA-I mutants to ECs -** Initially we compared the endothelial binding properties of WT apoA-I either isolated from plasma or produced as a recombinant protein. ECs were incubated with radiolabeled lipid-free WT apoA-I at 4°C in the absence (total) or presence of a 40-fold excess of unlabelled plasma WT apoA-I (non-specific). The difference between the total and the non-specific binding corresponds to the specific binding. The recombinant and plasma-derived WT isoproteins of apoA-I showed the same total and specific binding to ECs. Therefore, in further experiments, plasma WT apoA-I was used as a control to compare the behaviour of the different apoA-I mutants.

The apoA-I(Δ144-165) mutant showed similar total and specific binding to ECs as WT apoA-I. The two mutants with a defective carboxy-terminal sequence, apoA-I(Δ185-243) and apoA-I(L218A/L219A/V221A/L222A), showed 50% and 80% decreases in total and specific endothelial binding, respectively (figure 1). In contrast, apoA-I(Δ1-59) and apoA-I(Δ1-59/Δ185-243), showed a dramatic 25-fold increase in total binding. The binding of apoA-I(Δ1-59) could not be competed by unlabelled WT apoA-I so that no specific binding could be recorded (figure 1). The calculated specific binding of apoA-I(Δ1-59/Δ185-243) did not differ from that of WT apoA-I .

We repeated these experiments at 37°C where ligands are not only bound but also internalized by ECs (16,32) and therefore the absolute amount of cell associated radioactivity is higher. In principle we made the same observations as described for binding at 4°C: WT apoA-I from plasma, recombinant WT apoA-I, and apoA-I(Δ144-165) did not differ from each other whereas the apoA-I(Δ185-243) and apoA-I(L218A/L219A/V221A/L222A) mutants showed strongly reduced total and specific cell association and the apoA-I(Δ1-59) and apoA-I(Δ1-59/Δ185-243) mutants showed massively increased total cell association which could not be competed by WT apoA-I (data not shown).

**Transport of lipid-free apoA-I mutants through ECs -** Next, we analysed the transport of the different apoA-I mutants though ECs cultivated in a transwell system. Radiolabeled lipid-free WT or mutant apoA-I was added to the apical side with or without 40-fold excess of unlabelled competitor (WT apoA-I). After 1 h incubation at 37°C, the medium of the basolateral compartment was collected to measure radioactivity. The specific transport was calculated as the difference between total transport (radioactivity after incubation without competitor) and non-specific transport (radioactivity after incubation with competitor). As shown in figure 2, specific transports of plasma WT apoA-I, recombinant WT apoA-I,

and apoA-I(Δ144-165) were similar. In contrast, the specific transports of apoA-I(Δ185-243) and apoA-I(L218A/L219A/V221A/L222A) were decreased by 90%. No specific transports could be calculated for apoA-I(Δ1-59) and apoA-I(Δ1-59/Δ185-243) mutants because both in the presence or absence of the competitor the same amounts of radioactivity were recovered in the basolateral compartment of the transwell cell culture dish.

**ApoA-I with mutations in the carboxy-terminus is not lipidated after transendothelial transport -** In previous studies we observed a change in particle size and electrophoretic mobility of lipid-free WT apoA-I after the transport through ECs which we interpreted as a result of lipidation (16-17). Therefore, we compared the particle sizes of WT apoA-I and the dysfunctional apoA-I(L218A/L219A/V221A/L222A) mutant before and after transport through ECs (figure 3). After transport and recovery from the basolateral compartment WT apoA-I was fractionated by gel filtration into two peaks, one peak with identical elution volume (87.6 ml +/- 0.5 ml) and hence size of the starting material and one new peak with lower elution volume (72.6 ml +/- 0.5 ml) and hence larger particle size (figure 3A). By contrast, the comparison of the gel filtration profiles of the binding- and transport-defective apoA-I(L218A/L219A/V221A/L222A) mutant before and after incubation with ECs did not reveal the occurrence of any new peak (figure 3B). Already before incubation with cells and hence in the lipid-free state, this mutant was eluted in two peaks, one with the size of WT apoA-I (87.6 ml +/- 0.5 ml) and one corresponding to larger particle size (77.1 ml +/- 0.5 ml). The larger sized fraction of apoA-I(L218A/L219A/V221A/L222A) has a higher elution volume than the fraction formed after transport of WT apoA-I and did not change after transport. We therefore assume that this fraction represents lipid-free aggregates of apoA-I(L218A/L219A/V221A/L222A).

**Role of ABCA1, ABCG1, and SR-BI for binding, cell association, and transport of lipid-free WT apoA-I -** Using specific siRNAs (15), we investigated the effects of ABCA1, ABCG1, and SR-BI knock-down alone and the silencing of both ABCA1 and ABCG1 together on endothelial binding, cell association, and transport of lipid-free WT apoA-I (figure 4). ABCA1, ABCG1, and SR-BI transcription were reduced by about 80 – 90% in cells transfected with specific siRNA. The remaining protein expression of ABCA1, ABCG1, and SR-BI after silencing was approximately 50% as assessed by Western blotting and already shown previously (15,17). Also as reported previously, knock-down of ABCA1, but not ABCG1 or SR-BI reduced the binding of lipid-free WT apoA-I at 4°C (figure 4A). The knockdown of ABCA1 and ABCG1 together revealed the same reduction of apoA-I binding as the single knock-down of ABCA1 by about 60%. At 37°C, suppression of ABCA1 and ABCG1 either individually or both together but not the knock-down of SR-BI diminished the cell association (figure 4B) and transendothelial transport of initially lipid-free apoA-I (figure 4C) by about 40%.
These at first sight discrepant observations at 4°C and 37°C could be explained by a previously proposed two-step model in which ABCA1-mediated lipid-efflux generates a lipidated particle that secondarily interacts with ABCG1 (18-20). To test this hypothesis we analysed the binding, association and transport of HDL reconstituted artificially with WT or mutant apoA-I.

**Interactions of rHDL with ECs –** We first compared the binding (at 4°C) and association properties (at 37°C) of WT apoA-I in either the lipid-free or pre-lipidiated forms. The binding of lipid-free apoA-I was competed by rHDL to similar degree as by lipid-free apoA-I itself or native HDL (figure 5A). The binding of rHDL was not competed by lipid-free apoA-I but by both reconstituted and native HDL (figure 5A). Cell association experiments yielded similar findings. Endothelial cell association of lipid-free apoA-I was competed with 40-fold excesses of unlabeled apoA-I, lipidated apoA-I, or HDL to similar degree (figure 5B). Cell association of rHDL was competed with an excess of either rHDL or native HDL (figure 5B). By contrast to the binding experiment, we however observed that also lipid-free apoA-I competed the cell association of rHDL, although to less extent (about 35%) than rHDL or native HDL (about 60%). These observations provide further evidence that at 37°C the lipidation of apoA-I by ABCA1 generates a lipidated particle which can then compete the cellular interaction of pre-lipidated apoA-I.

**Binding and transport of reconstituted HDL containing WT apoA-I or apoA-I(L218A/L219A/V221A/L222A) –** Next, we

exploited the apoA-I(L218A/L219A/V221A/L222A) mutant with defects in ABCA1-mediated lipid-efflux as well as specific endothelial binding and transport to test the hypothesis that transendothelial transport of lipid-free apoA-I occurs by a two-step-mechanism where apoA-I is first lipidated by ABCA1-dependent lipid-efflux to then undergo ABCA1-independent transport through ECs. We first lipidated WT apoA-I or apoA-I(L218A/L219A/V221A/L222A). The lipidation and resulting particle formation was verified by native agarose gel electrophoresis and electron microscopy. Both WT apoA-I and apoA-I(L218A/L219A/V221A/L222A) formed particles which had a higher electrophoretic mobility than the respective lipid-free apolipoproteins. rHDL containing apoA-I(L218A/L219A/V221A/L222A) were slightly less negatively charged than rHDL containing WT apoA-I (figure 6A). In addition, the Sudan black staining of the agarose gel, clearly reveals the lipidation of both WT apoA-I and apoA-I(L218A/L219A/V221A/L222A). After lipid staining the bands containing rHDL with WT or mutant apoA-I were similarly intense (figure 6B). Electron microscopy revealed that both WT apoA-I and apoA-I(L218A/L219A/V221A/ L222A) formed discoidal particles (figure 6C).
We then used the pre-lipidated particles to perform binding and transport studies. Neither the specific binding (figure 6D) nor the specific transport (figure 6E) differed between particles containing either WT apoA-I or the mutant apoA-I(L218A/L219A/V221A/L222A). Thus pre-lipidation can overcome the binding and transport defects of the dysfunctional apoA-I(L218A/L219A/V221A/L222A) mutant. These findings corroborate the hypothesis that lipidation of apoA-I is necessary for specific transcytosis.

**Binding and transport of reconstituted HDL containing apoA-I(Δ1-59) and apoA-I(Δ1-59/Δ185-243) -** We then investigated the effects of prior cell-free lipidation on binding and transport of the two apoA-I mutants which in the lipid-free form showed excessive non-specific endothelial binding and transport, namely apoA-I(Δ1-59) and apoA-I(Δ1-59/Δ185-243). After cholate dialysis with POPC in a molar ratio of 1:40, both mutant rHDL particles showed higher electrophoretic mobility than the respective lipid-free apolipoproteins (figure 7A). The efficacy of lipidation was further confirmed by lipid staining of the agarose gel (figure 7B). The two mutant rHDL particles however differed from each other and from normal rHDL by electrophoretic mobility (figures 7A and 7B). Both mutants formed discoidal particles (figure 7C). With these lipidated mutants we performed binding and transport studies. We used native HDL as the competitor, because reconstituted and native HDL competed equivalently (see figure 5). As shown in figure 7D, specific binding of the lipidated WT apoA-I was about 60 % of the specific binding of lipid-free WT apoA-I. However, pre-lipidated apoA-I(Δ1-59) and pre-lipidated apoA-I(Δ1-59/Δ185-243) showed 4-fold and 8-fold higher total binding than pre-lipidated WT apoA-I. In contrast to lipidated WT apoA-I, it was not possible to compete this binding with excess HDL indicating that these mutants keep their very high non-specific endothelial binding properties also after pre-lipidation. In contrast to apoA-I(L218A/L219A/V221A/L222A), pre-lipidation of either apoA-I(Δ1-59) or apoA-I(Δ1-59/Δ185-243) did not rescue their defective specific transendothelial transport (figure 7E). These findings suggest that lipidation of initially lipid-free apoA-I is not sufficient for the specific transport through ECs of the apoA-I(Δ1-59) or apoA-I(Δ1-59/Δ185-243) mutants.

**Role of ABCA1, ABCG1, and SR-BI for transport of rHDL -** To analyze which of the known apoA-I/HDL binding proteins are participating in the transport of pre-lipidated apoA-I we used siRNAs to suppress ABCA1, ABCG1 or SR-BI. Knock down of ABCG1 and SR-BI but not of ABCA1 decreased the specific transendothelial transport of pre-lipidated WT apoA-I (figure 8A) and pre-lipidated apoA-I(L218A/L219A/V221A/L222A) (figure 8B). The reduction of the transport capacity of lipidated apoA-I(L218A/L219A/V221A/L222A) by knock down of ABCG1 or SR-BI was smaller (-44% +/-7 %) compared to lipidated WT apoA-I (-60% +/- 15%).

## Discussion

We recently provided several arguments that the transendothelial transport of apoA-I and HDL occurs by specific transport rather than unspecific filtration: 1) a considerable proportion of apoA-I and HDL transport is temperature sensitive and can be competed by excess of apoA-I and HDL, respectively, but not with albumin or LDL (15-16). 2) The specific fraction of transendothelial apoA-I transport can be

inhibited by knock-down of ABCA1 and leads to the secretion of lipidated particles (17). 3) The specific fraction of transendothelial HDL transport can be reduced by knock-down of ABCG1 or SR-BI and leads to the secretion of an HDL particle of reduced size (15). The findings of this study further support the specificity of transendothelial apoA-I and HDL transport by stringent structure-function-relationships for apoA-I that reveals the importance of the carboxy-terminal apoA-I domain for this process:

1. Both the deletion of the carboxy-terminus of apoA-I as well as amino acid substitutions within the carboxy-terminus of apoA-I nearly abolished the specific endothelial binding and transendothelial transport of apoA-I (figure 1, 2). The deletion of the carboxy-terminus was previously shown to be defective in inducing ABCA1-mediated phospholipid and cholesterol efflux from macrophages and to form nascent and mature HDL particles in vivo (22,26,33). Also in our endothelial transwell cell culture model both apoA-I(Δ185–243) and apoA-I(L218A/L219A/V221A/L222A) failed to form HDL-like particles although only the specific but not the non-specific fraction of transendothelial transport was abolished.

2. The deletion of the amino-terminus of apoA-I, alone or together with the carboxy-terminus, tremendously increased the non-specific binding of apoA-I(Δ1-59) and apoA-I(Δ1-59/Δ185-243), respectively, and interfered with the specific transport of these mutants both in the lipid-free and lipidated forms (figure 1, 2, 7). Interestingly, these mutants were previously found to elicit normal or only moderately decreased ABCA1-dependent lipid efflux from macrophages and similar mutations (apoA-I(Δ1-41) and apoA-I(Δ1-41/Δ185-243)) promoted biogenesis of HDL particles in vivo (22). However, in the large background of excessive cellular non-specific binding to cells, we may have overlooked specific components of transendothelial transport. In fact, the double deletion mutant (apoA-I(Δ1-59/Δ185-243) but not the amino-terminal deletion apoA-I(Δ1-59) mutant showed more than normal specific binding to ECs (figure 1). Alternatively, the deletion of the amino-terminus and especially both the amino-terminus and carboxy-terminus may expose a midregional domain of prototypic anti-parallel amphipathic alpha-helices which has a very high affinity to lipids (34). According to a model proposed by Phillips et al. this central domain of apoA-I may bind very efficiently and solubilise lipids of plasma membranes which are generated by ABCA1 (35). In fact, pretreatment of ECs with cyclosporine A, which was shown by us and others to trap dysfunctional ABCA1 on the cells surface (17,36), reduced the excessive non-specific binding of apoA-I(Δ1-59) and apoA-I(Δ1-59/Δ185-243) (data not shown). Furthermore, it has been shown that the amino-terminal deletion destabilizes apoA-I and leads to unfolding of the alpha-helices in the carboxy-terminal domain which is responsible for specific interactions with ABCA1 (37-38).

3. The deletion of a central domain in apoA-I(Δ144-165) interfered neither with specific binding nor specific transendothelial transport (figure 1, 2). This mutant was previously found to behave like WT apoA-I in mediating ABCA1 dependent lipid efflux but to be defective in lecithin:cholesterol acyl transferase (LCAT) activation (22,39-40).

Taken together our data further emphasize the importance of ABCA1 as a rate limiting step for transendothelial apoA-I transport. Several authors have provided evidence for a physical interaction of apoA-I with ABCA1 (22,41). The formation of a high affinity complex of apoA-I with ABCA1 is thought to play an important regulatory first step in ABCA1-mediated lipid efflux by stabilizing ABCA1 in the plasma membrane and eliciting signalling events which enrich distinct plasma membranes with lipids for facilitated removal by apoA-I (39,42-43). However, the signalling events elicited by apoA-I/ABCA1 interaction have also been related to other cellular responses such as cell migration and endocytosis (44-45). As the consequence, the defective transendothelial transport of apoA-I mutants with a missing or dysfunctional carboxy-terminal ABCA1-interaction domain may be principally explained by disturbances in different downstream events. Our present results shed some light into these different scenarios:
By using gel filtration we here corroborated our previous finding that transendothelial transport leads to the secretion of a lipidated particle on the basolateral side (16). The apoA-I(L218A/L219A/V221A/L222A) mutant, which shows strongly reduced specific but normal non-specific transendothelial transport (data not shown), was not recovered as a lipidated particle (figure 3). However, after prior cell-free lipidation the carboxy-terminal apoA-I mutant

was normally bound and transported by ECs (figure 6). This finding suggests that the transendothelial transport of apoA-I is initiated by ABCA1-mediated lipidation of apoA-I and followed by ABCA1-independent transport steps. These downstream pathways may be shared with the transport of HDL, since knock-down of ABCG1 or SR-BI inhibited the specific transport of native HDL (15) as well as rHDL containing either WT apoA-I or apoA-I(L218A/L219A/V221A/L222A) (figure 8). In agreement with normal binding of rHDL containing apoA-I(L218A/L219A/V221A/L222A) ldlA-7 cells expressing SR-BI were previously found to bind rHDL containing apoA-I(Δ185-243) with similar affinity as rHDL with WT apoA-I (46). In general our findings resemble similar findings and models on the interaction of ABCA1, ABCG1, and SR-BI in cholesterol efflux: ABCA1-mediated lipid efflux to initially lipid-free apoA-I generates particles which then interact with ABCG1 and SR-BI for enhanced cholesterol efflux (18-20).

Interestingly, the lipidation did not restore the abnormal binding and transport of apoA-I(Δ1-59) or apoA-I(Δ1-59/Δ185-243 (figure 7). Although less intense than in the lipid-free form, also in the pre-lipidated form these mutants showed strongly enhanced binding to ECs and not-recordable specific binding and transendothelial transport. This suggests that the amino-terminal domain is an important structural determinant for specific endothelial binding and transendothelial transport of HDL. Previously, rHDL containing apoA-I(Δ1-59/Δ185-243) were reported to be unable to compete for rHDL binding to ldlA-7 cells expressing the murine SR-BI receptor (27). Indirectly, this supports the importance of SR-BI as a rate limiting factor for endothelial binding and transport of HDL. However, rHDL containing the apoA-I(Δ1-59) mutant, which in our hands is also defective in endothelial binding and transport, was reported to bind with normal affinity to SR-BI overexpressing ldlA-7 cells (27,46). The reason for this discrepancy remains unclear.

In summary, the distinct binding and transport defects of apoA-I mutants provide further support that transendothelial transport of apoA-I and HDL requires defined structural domains and hence involves specific protein/protein interactions rather than unspecific filtration. These experiments also support the importance of ABCA1 for the transport of lipid-free apoA-I and provided first hints on the underlying mechanisms: By lipidating apoA-I ABCA1 helps to generate a particle which is then processed by ABCA1-independent mechanisms for transendothelial transport. Like the transport of mature HDL, the processing of these nascent HDL particles appears to involve ABCG1 and SR-BI.

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## Footnotes

The abbreviations used are: HDL, high density lipoprotein; apoA-I, apolipoprotein A-I; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; SR-BI, scavenger receptor BI; WT, wild-type; ECs, aortic endothelial cells; rHDL, reconstituted HDL

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## Figure Legends

Figure 1. **Binding of WT and mutant apoA-I to ECs.** Cells were cultured in 12- well dishes for 48 hours. Then, after pre-chilling on ice, the cells were incubated with 5 µg/ml of the indicated WT or mutant $^{125}$I-apoA-I isoform in the absence (total) or presence (non-specific) of a 40-fold excess of unlabeled WT apoA-I. After 2 hours of incubation at 4°C, the specific binding was determined by subtracting the values of non-specific binding from those of total binding. The results are represented as means ± SD of at least 3 individual experiments.

Figure 2. **Transport of WT and mutant apoA-I through ECs.** Cells were cultured on membrane inserts 48 hours before the assay. 5 µg/ml of the indicated WT or mutant $^{125}$I-apoA-I isoform as well as no or a 40-fold excess of unlabeled WT apoA-I were added to the apical compartment. The media of the basolateral compartments of the transwell chambers were collected after incubation for 1 hour at 37°C to measure the radioactivity. Specific transport from the apical to the basolateral compartment was calculated as the difference in radioactivity between the samples with (non-specific transport) and without (total transport) excess of WT apoA-I. The results are represented as means ± SD of at least 3 individual experiments.

Figure 3. **Particle size of WT apoA-I and apoA-I(L218A/L219A/V221A/L222A) before and after transport through ECs.** The transport experiment was performed as described in figure 3, however only in the absence of excess unlabeled apoA-I. Both lipid-free apoA-I not incubated with cells and the material in the basolateral compartment were fractionated by gel filtration. (A) WT $^{125}$I-apoA-I, (B) $^{125}$I-apoA-I(L218A/L219A/V221A/L222A).

Figure 4. **Role of ABCA1, ABCG1, and SR-BI for binding, cell association, and transport of WT apoA-I.** ECs were transfected with specific siRNA against ABCA1, ABCG1, SR-BI, not coding siRNA and mock (not transfected cells). 65 – 72 hours after transfection, binding (A), cell association (B), and transport assays (C) were performed as described above. *P<0.05 **P<0.01, ***P<0.001, ns not significantly different compared to not transfected cells.

Figure 5. **Cross-competition of lipid-free and lipidated apoA-I.** Binding (A) and cell association (B) assays were performed. ECs were incubated with 5 μg/ml of $^{125}$I-apoA-I or $^{125}$I-rHDL containing WT apoA-I for 2 hours at 4°C (binding) or for 1 hour at 37°C (cell association) in the absence (total) or presence of a 40-fold excess of the indicated competitor.

Figure 6. **Binding and transport of reconstituted HDL (rHDL) containing WT apoA-I and apoA-I(L218A/L219A/V221A/L222A).** The lipidation of the rHDL particles was performed by the cholate dialysis method and the particle formation was analysed by native agarose gel electrophoresis stained with Coomassie for proteins (A) and with Sudan black for lipids (B). Electron microscopy of the formed particles was performed (C). (D) Specific binding at 4°C of 10 μg/ml $^{125}$I-rHDL particles containing either WT apoA-I or apoA-I(L218A/L219A/V221A/L222A). (E) Specific Transport of 10 μg/ml $^{125}$I-rHDL particles containing either WT apoA-I or apoA-I(L218A/L219A/V221A/L222A) through a monolayer of ECs.

Figure 7. **Binding and transport of rHDL containing WT apoA-I, apoA-I(Δ1-59), and apoA-I(Δ1-59/Δ185-243)**. Lipidation was verified native agarose gel electrophoresis stained with Coomassie for proteins (A) and with Sudan black for lipids (B) and by electron microscopy (C). (D) Binding at 4°C of 10 μg/ml $^{125}$I-rHDL containing WT apoA-I, apoA-I(Δ1-59), or apoA-I(Δ1-59/Δ185-243). (E) Specific transport through a monolayer of ECs of 10 μg/ml $^{125}$I rHDL containing WT apoA-I, apoA-I(Δ1-59), or apoA-I(Δ1-59/Δ185-243).

Figure 8. **Role of ABCA1, ABCG1, and SR-BI in the transport of rHDL containing WT apoA-I or apoA-I(L218A/L219A/V221A/L222A)**. ECs were transfected with siRNA coding for ABCA1, ABCG1, SR-BI and not coding siRNA. 65 – 72 hours after transfection transport assays were performed. ECs were incubated with 10 μg/ml of $^{125}$I-rHDL for 1 hour in the absence or presence of a 40-fold excess of unlabeled rHDL. Specific transport was calculated by subtracting the values of non-specific transport from those of total transport. (A) Specific transport of rHDL containing WT apoA-I. (B) Specific transport of rHDL containing apoA-I(L218A/L219A/V221A/L222A). **P<0.01, ***P<0.001, ns not significantly different compared to not transfected cells.

Figure 1

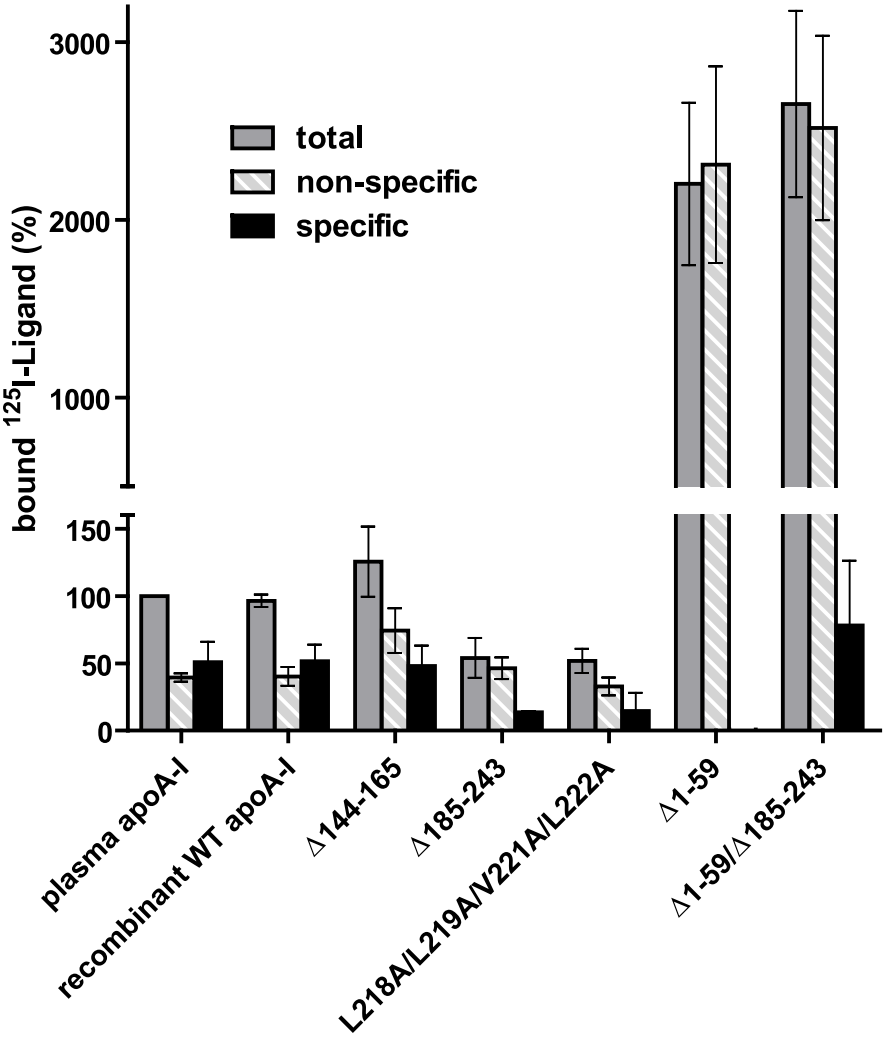


Figure 2

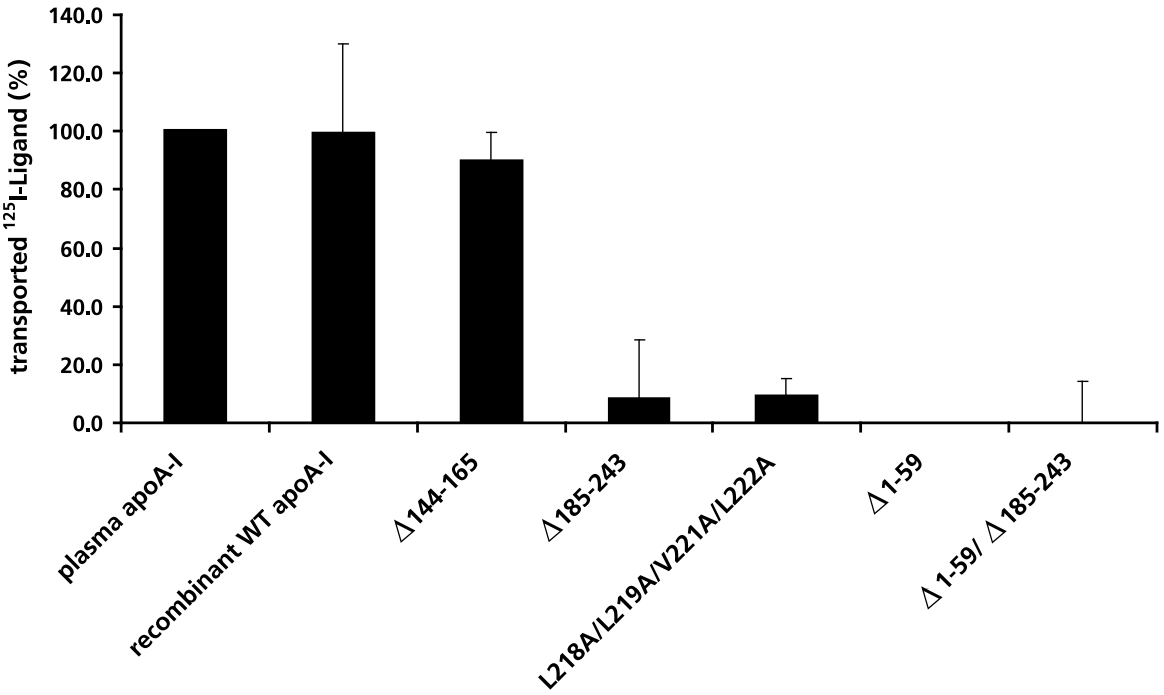
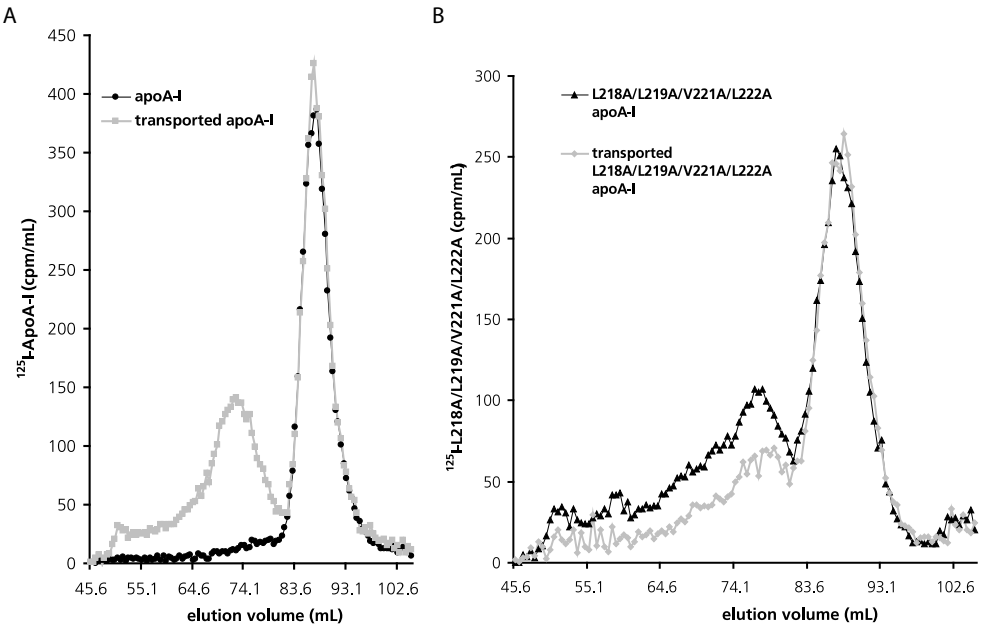


Figure 3



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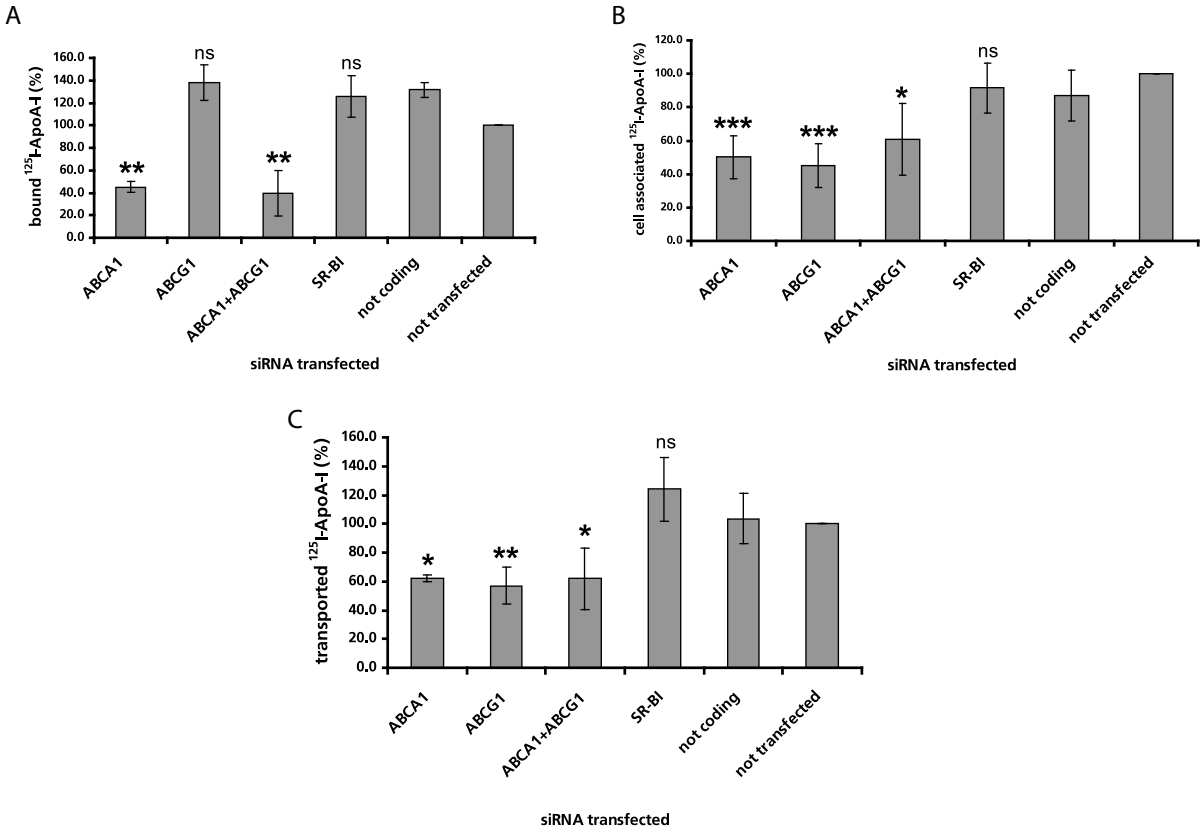

Figure 5

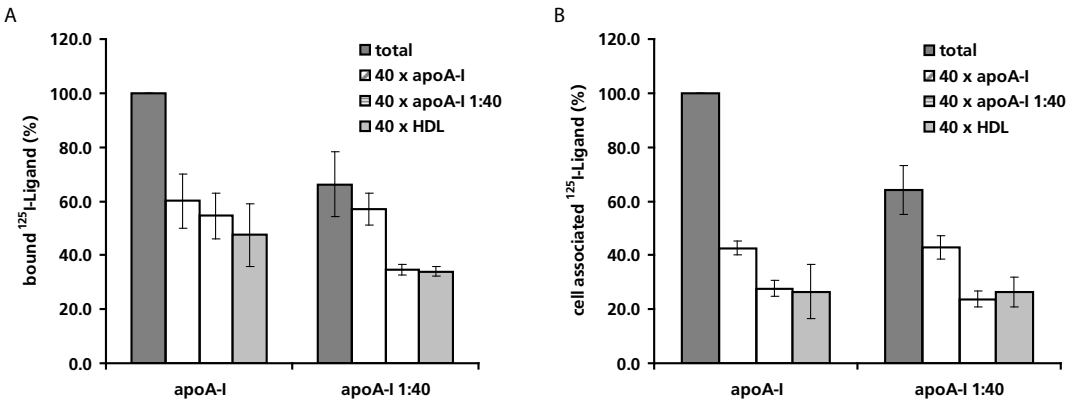

Figure 6

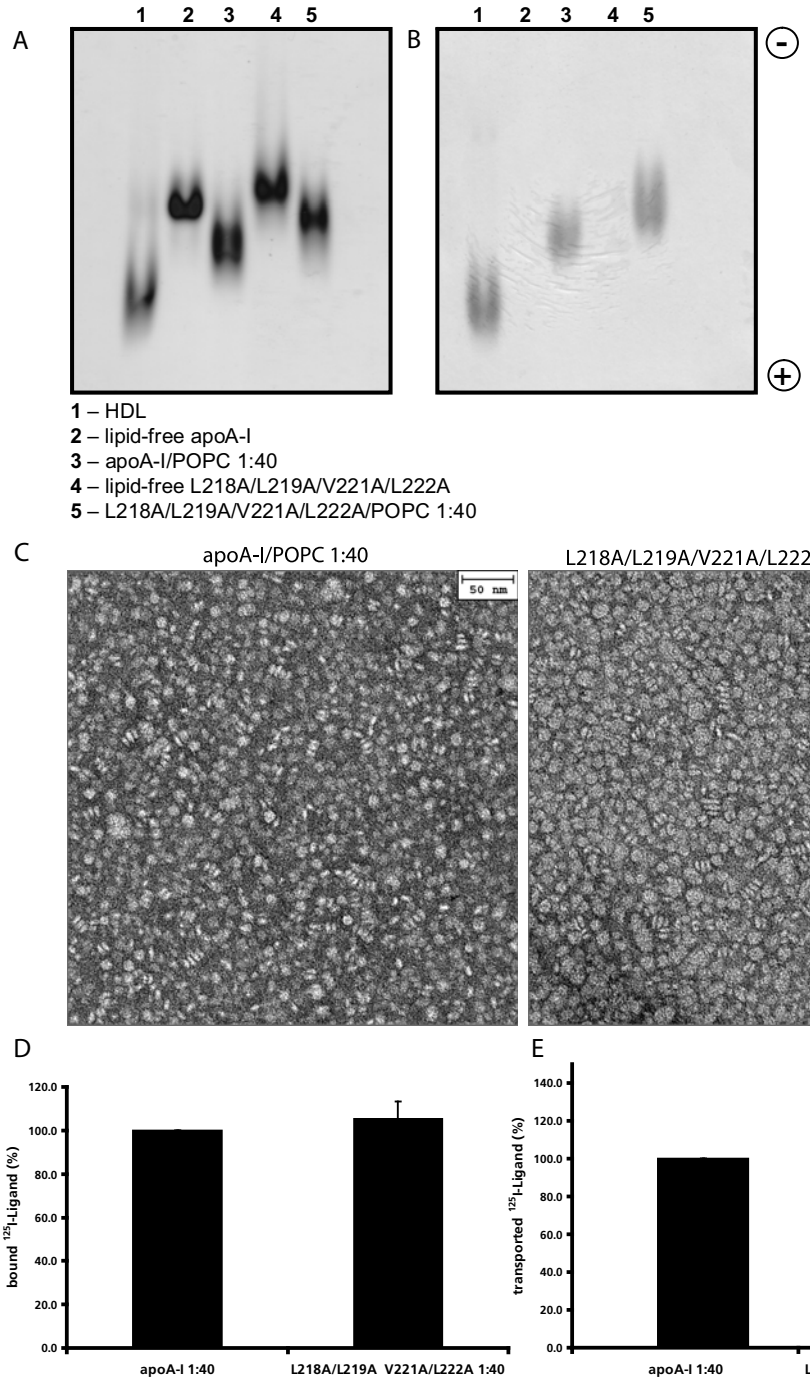

Figure 7

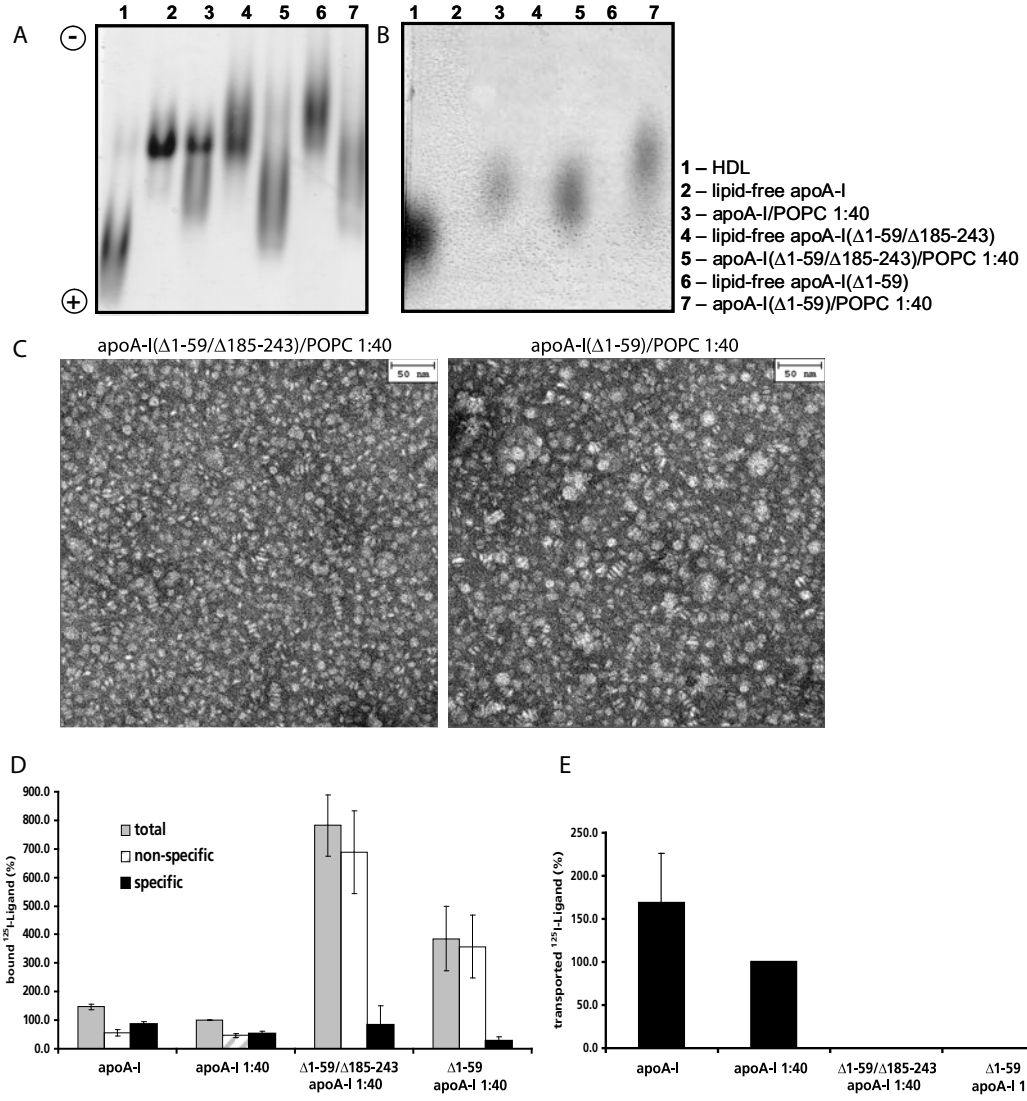

Figure 8